Roles of the Yap1 Transcription Factor and Antioxidants in *Saccharomyces cerevisiae*'s Tolerance to Furfural and 5-Hydroxymethylfurfural, Which Function as Thiol-Reactive Electrophiles Generating Oxidative Stress

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Development of the tolerance of *Saccharomyces cerevisiae* strains to furfural and 5-hydroxymethylfurfural (HMF) is an important issue for cellulosic ethanol production. Although furfural and HMF are known to induce oxidative stress, the underlying mechanisms are largely unknown. In this study, we show that both furfural and HMF act as thiol-reactive electrophiles, thus directly activating the Yap1 transcription factor via the H_2O_2-independent pathway, depleting cellular glutathione (GSH) levels, and accumulating reactive oxygen species in *Saccharomyces cerevisiae*. However, furfural showed higher reactivity than did HMF toward GSH *in vitro* and *in vivo*. In line with such toxic mechanisms, overexpression of $YAP1^{C620F}$, a constitutively active mutant of *YAP1*, and Yap1 target genes encoding catalases (*CTA1* and *CTT1*) increased tolerance to furfural and HMF. However, increasing GSH levels by overexpression of genes for GSH biosynthesis (*GSH1* and *GLR1*) or by the exogenous addition of GSH to the culture medium enhanced tolerance to furfural but not to HMF.

Bioethanol production from lignocellulosic biomass is currently one of the key subjects of the biofuel industry. However, because of the extremely rigid and complex nature of lignocellulose, pretreatment is required to make this material available for subsequent enzymatic digestion and microbial fermentation [1]. The commonly used dilute acid pretreatment generates numerous chemical by-products such as furan aldehydes, weak acids, and phenol derivatives, which inhibit microbial cell growth and ethanol fermentation [2]. To overcome the inhibitory effects of these compounds, various physical and chemical methods of medium detoxification have been developed [1]. However, because of the high cost of medium detoxification methods, biodetoxification of inhibitors by using tolerant microorganisms is considered a more practical alternative [2].

Furfural and 5-hydroxymethylfurfural (HMF) are derived from the dehydration of pentoses and hexoses, respectively [3]. The amounts of furfural and HMF generated during pretreatment are variable depending on the types of raw materials and pretreatment methods. In previous studies, up to 26 mM furfural and 47 mM HMF were detected in the dilute acid hydrolysates [4]. These furan aldehydes are known as the most potent inhibitors of microbial cell growth [4, 5]. The toxic aldehyde groups of furfural and HMF can be reduced to hydroxyl groups by several oxidoreductases, including alcohol dehydrogenases (Adh1, Adh6, and Adh7) [6–8], aldehyde reductase (Ari1) [9], and methylglyoxal reductases (Gre2 and Gre3) in *Saccharomyces cerevisiae* cells [8, 10–13] and aldehyde dehydrogenase (YqhD) and methylglyoxal reductase (DkgA) in *Escherichia coli* [14]. These enzymes consume NADH and NADPH as cofactors during the reduction process. On the other hand, furfural and HMF can also be detoxified by oxidation by Ald6 aldehyde dehydrogenase [13]. In line with these detoxification mechanisms, furfural- and/or HMF-tolerant yeast strains could be generated by the overexpression of *ADH6*, *ADH7*, and *ALD6* [6, 7, 13]. In addition, overexpression of *ZWF1*, involved in NADPH regeneration through a pentose phosphate pathway, also enhanced furfural tolerance [15].

In order to develop strains tolerant to furan aldehydes, it is important to understand the cellular toxic mechanisms of furan aldehydes. Genome-wide transcriptome analyses have revealed a wide range of cellular functions regulated by furfural and HMF in yeast [16, 17]. Because different yeast strains and conditions were used for these previous experiments, little overlap exists among the genes identified to be upregulated by furfural and HMF. However, induction of stress-responsive genes was commonly observed when cells were treated with either furfural or HMF. Especially, Yap1, a major oxidative stress regulator, was identified as one of the key regulators involved in genomic adaptation to HMF in *S. cerevisiae*, suggesting that HMF might elicit oxidative stress [17, 18]. In agreement with the role for Yap1 in adaptation to HMF, the overexpression of *YAP1* has been shown to increase tolerance to HMF and dilute acid spruce hydrolysates [18]. Furthermore, furfural has been shown to induce the accumulation of reactive oxygen species (ROS), leading to damage in cell components, including mitochondrial and vacuolar membranes, and chromatin in yeast [19]. However, how furfural and HMF induce oxidative stress has not yet been elucidated. In addition, the inhibition of biosynthetic pathways by depletion of NADPH during the reduction of furfural and HMF might also be responsible for their toxicity. In *E. coli*, the NADPH-dependent sulfur assimilation pathway was shown to be inhibited in furfural-treated cells, and supplementation with sulfur-containing amino acids, cys-

teine and methionine, could increase furfural tolerance (20). Although furfural and HMF share the same toxic aldehyde group, furfural has higher toxicity than does HMF (21, 22). However, detailed mechanisms for the differences in toxicity between furfural and HMF have not yet been clarified.

In this study, as an effort to develop strains tolerant to furfural and HMF, we investigated the mechanisms by which furfural and HMF induce oxidative stress. We have demonstrated that thiol reactivity of furfural and HMF contributes to inducing oxidative stress, but furfural has higher thiol reactivity than does HMF. Based on the identified mechanisms, we generated yeast strains tolerant to HMF and/or furfural.

MATERIALS AND METHODS

Yeast strains, media, and growth conditions. *S. cerevisiae* BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was used in this study. BY4741-derived *gpx3Δ* (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 gpx3Δ::kanMX4*) and *hgt1Δ* (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hgt1Δ::kanMX4*) deletion mutants were generously provided by W.-K. Huh (Seoul National University, South Korea). Yeast cells were grown in YPD medium (2% dextrose, 1% yeast extract, and 2% Bacto peptone) or synthetic complete (SC) medium (2% dextrose, 0.67% yeast nitrogen base without amino acids, and 0.2% amino acid dropout mixture) lacking appropriate components for plasmid selection. Cells were grown overnight at 30°C in a 96-well deep-well plate and reinoculated to an A_{600} of 0.01 in medium containing 20 to 40 mM furfural (Sigma-Aldrich, USA) or 30 to 40 mM HMF (Sigma-Aldrich, USA). Cell growth was monitored by using a 96-well plate spectrophotometer (Multiskan GO; Thermo Scientific, USA).

Construction of plasmids. *YAP1* and *ZWF1* open reading frames (ORFs) were amplified by PCR and cloned into the XbaI and XhoI sites of a pRS415ADH vector. The *YAP1^C620F^* mutant was generated by overlapping PCR and cloned into the XbaI and XhoI sites of pRS415ADH. PCR-amplified *CTA1* and *CTT1* ORFs were cloned into the HindIII and XhoI sites of pRS416ADH, and *GSH1*, *GSH2*, and *GLR1* ORFs were cloned into the XbaI and XhoI sites of pRS416ADH.

qRT-PCR analysis. Cells were grown in YPD medium to an A_{600} of 1 and then treated with 30 mM HMF, 20 mM furfural, or 0.4 mM H_2O_2. Total RNA was isolated from yeast cells, and the relative amount of specific mRNA was determined by quantitative reverse transcription-PCR (qRT-PCR) (23). Briefly, 1 mg of the total RNA was subjected to reverse transcription in a 20-μl reaction mixture containing 200 U of myeloblastosis virus reverse transcriptase (M-biotech, South Korea) and 0.1 mg of oligo(dT) at 42°C for 1 h. Quantitative PCR (qPCR) was performed with a LightCycler 480 II instrument (Roche Diagnostics, Germany) using the SYBR green PCR Master Mix (Roche Diagnostics, Germany) and gene-specific primers (*YAP1*, 5′-ATGATGTCGTTCCATCTAAGGAAGG-3′ and 5′-CAACCCCTCTTTCTGAACATTTTGC-3′; *TRX2*, 5′-AAAGTTT GCAGAACAATATTCTGACG-3′ and 5′-TTGGCACCGACGACTCTG GTAACC-3′; *MET16*, 5′-AACAGTATATAGATGCAAACAATGTAC-3′ and 5′-CACACTCGGTCTTGGCCTTGCCC-3′; *ACT1*, 5′-GCCGAAAG AATGCAAAAGGA-3′ and 5′-TAGAACCACCAATCCAGACGG-3′) under the following conditions: 95°C for 5 min, followed by 45 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 20 s, and cooling to 40°C for 30 s. All experiments were performed in triplicate, and the data were normalized using the *ACT1* gene as a control.

Measurement of intracellular ROS using fluorescence-activated cell sorter (FACS) analysis. Levels of intracellular ROS were measured using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) (Sigma-Aldrich, USA) (24). Cells exponentially grown in YPD medium were treated with 20 mM furfural; 15, 30, or 45 mM HMF; or 5 mM H_2O_2 for 8 h, and then H_2DCF-DA was added to a final concentration of 10 μg/liter and incubated for 60 min at 30°C. The cells were washed twice in phosphate-buffered saline (PBS) and were analyzed using flow cytometry. The fluorescence of cells from each sample was determined by using a FACSCanto

flow cytometer (Becton, Dickinson, USA) equipped with a 488-nm blue laser. Among the 10,000 cells analyzed for each sample, those cells with fluorescence intensities ranging from 10^2 to 10^4 were counted.

Measurement of GSH levels *in vitro*. The concentration of sulfhydryl groups of glutathione (GSH) was measured using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (Sigma-Aldrich, USA) (25). GSH (5 mM; Calbiochem, USA) was incubated with 30 or 60 mM furfural and 30 or 60 mM HMF in PBS buffer at 30°C. During the incubation, aliquots were taken and mixed with 150 μM DTNB in a buffer containing 30 mM Tris-HCl (pH 8.0) and 3 mM EDTA to 200 μl of total assay volume. Each sample was incubated for 5 min, and the color development was determined by using a 96-well plate spectrophotometer (Multiskan GO; Thermo Scientific, USA) at 412 nm.

Measurement of glutathione levels *in vivo*. Cells were grown in YPD medium to early exponential phase and treated with 30 mM HMF or furfural for 5 and 10 h. Harvested cells were washed, resuspended in cold 5% 5-sulfosalicylic acid in a 50 mM potassium phosphate buffer (pH 7.8) containing 3 mM EDTA, and disrupted with glass beads. The lysates were centrifuged (4°C, 13,200 × *g*) for 15 min, and the supernatant was obtained for the measurement of total and oxidized glutathione. Glutathione quantification was performed as described previously (26). For the estimation of total glutathione, 10 μl of 4 M triethanolamine was added to a 100-μl cell extract, and aliquots were taken and mixed with 150 μM DTNB in a buffer containing 50 mM potassium phosphate buffer (pH 7.8), 3 mM EDTA, 0.2 mM NADPH, and 0.25 U glutathione reductase (Sigma-Aldrich, USA) in 200 μl of a total assay volume. For the determination of oxidized glutathione disulfide (GSSG) levels, glutathione quantification assays were performed after eliminating GSH by incubating 2 μl of 1 M 2-vinylpyridine in 100-μl cell lysates for 1 h.

RESULTS

Furfural and HMF induce intracellular ROS accumulation in *S. cerevisiae*. In previous studies, it was shown that furfural induces ROS accumulation, resulting in damage to various cellular components in *S. cerevisiae* (19). Therefore, we investigated whether HMF can also exert the same ROS accumulation effect as furfural does. The cellular ROS levels were detected by incubating cells with 2′,7′-DCF diacetate, which is converted to a fluorescent compound upon ROS-dependent oxidation. 2′,7′-DCF diacetate is known to detect H_2O_2, peroxynitrite ($ONOO^-$), and hydroxyl radical (˙OH) (24). As a positive control, the cells were treated with 5 mM H_2O_2, and the fluorescent cells were counted by FACS analysis. In agreement with the previous report, ROS accumulation was detected after incubation of yeast cells with 30 mM furfural for 8 h (Fig. 1). Furthermore, treatment of HMF also induced ROS accumulation in a dose-dependent manner, indicating that furfural and HMF share an effect of inducing intracellular ROS accumulation (Fig. 1).

Furfural and HMF activate Yap1 as thiol-reactive electrophiles. Previous DNA microarray experiments have revealed that Yap1 is one of the key regulators involved in genomic adaptation to HMF in *S. cerevisiae* (17). Yap1 is activated by H_2O_2 and thiol-reactive electrophiles through different mechanisms (27). The H_2O_2-dependent activation of Yap1 is mediated by a glutathione peroxidase, Gpx3, which acts as an H_2O_2 sensor and induces intramolecular disulfide bond formation between N- and C-terminal Cys residues in Yap1. On the other hand, thiol-reactive electrophiles such as *N*-ethylmaleimide (NEM), acrolein, and 4-hydroxynonenal directly activate Yap1 by covalent modification of Cys residues in the C-terminal domain (28). Furthermore, H_2O_2 and thiol-reactive reagents have been shown to activate different subsets of Yap1 targets as well as common targets,
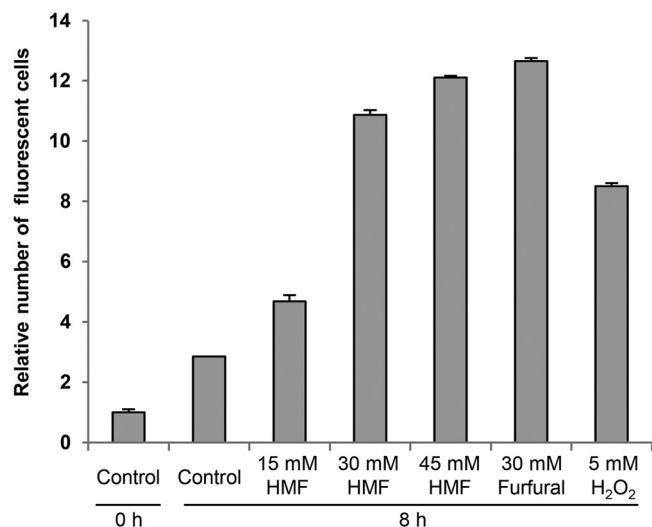
FIG 1 Both furfural and HMF induce ROS accumulation. Exponentially growing yeast cells were treated with the indicated amounts of HMF, furfural, or H_2O_2 for 8 h and treated with 2′,7′-DCF diacetate, a ROS indicator. The number of fluorescent cells was analyzed by FACS. Data represent an average of three experiments, and bars indicate plus standard deviations.

eliciting distinct adaptive responses (29). Therefore, we examined which pathway is responsible for the HMF-dependent activation of Yap1. In addition, we investigated whether furfural could also activate Yap1.

In order to understand the Yap1 activation pathway, we compared the expression levels of Yap1 targets, *TRX2*, *YAP1*, and *MET16*, in wild-type and *gpx3Δ* strains upon treatment with HMF or furfural (Fig. 2). The expression of *TRX2* and *YAP1* was induced when cells were treated with 0.4 mM H_2O_2, but their fold induction levels were dramatically reduced in the *gpx3Δ* strain, confirming the role for Gpx3 in the H_2O_2-dependent activation of Yap1 (Fig. 2A and B). The expression of *TRX2* and *YAP1* was also induced by 30 mM HMF and 20 mM furfural, although the fold induction levels were lower than those of H_2O_2-dependent induction. However, HMF- and furfural-dependent induction levels of *TRX2* and *YAP1* were largely unaffected by the lack of *GPX3*, suggesting that H_2O_2 might play a minimal role in the HMF- and furfural-dependent Yap1 activation pathways. Therefore, HMF and furfural might directly activate Yap1 as thiol-reactive electrophiles.

Unlike *TRX2* and *YAP1*, *MET16* is known to be responsive to thiol-reactive reagents rather than H_2O_2 (29). Accordingly, *MET16* was less sensitive to H_2O_2-dependent induction than were *TRX2* and *YAP1* (Fig. 2C). Moreover, HMF and furfural were more effective than H_2O_2 for the induction of *MET16*, further supporting the notion that HMF and furfural directly activate Yap1 as thiol-reactive reagents (Fig. 2C). Although HMF and furfural induce the accumulation of ROS, which include H_2O_2, Yap1 seems to be directly activated by HMF and furfural before the accumulation of H_2O_2.

Furfural has higher reactivity than does HMF toward GSH *in vitro* and *in vivo*. If HMF and furfural act as thiol-reactive electrophiles, they might react not only with Yap1 but also with other cellular sulfhydryl groups present in proteins and small molecular thiols such as GSH. GSH, the most abundant thiol in cells, serves
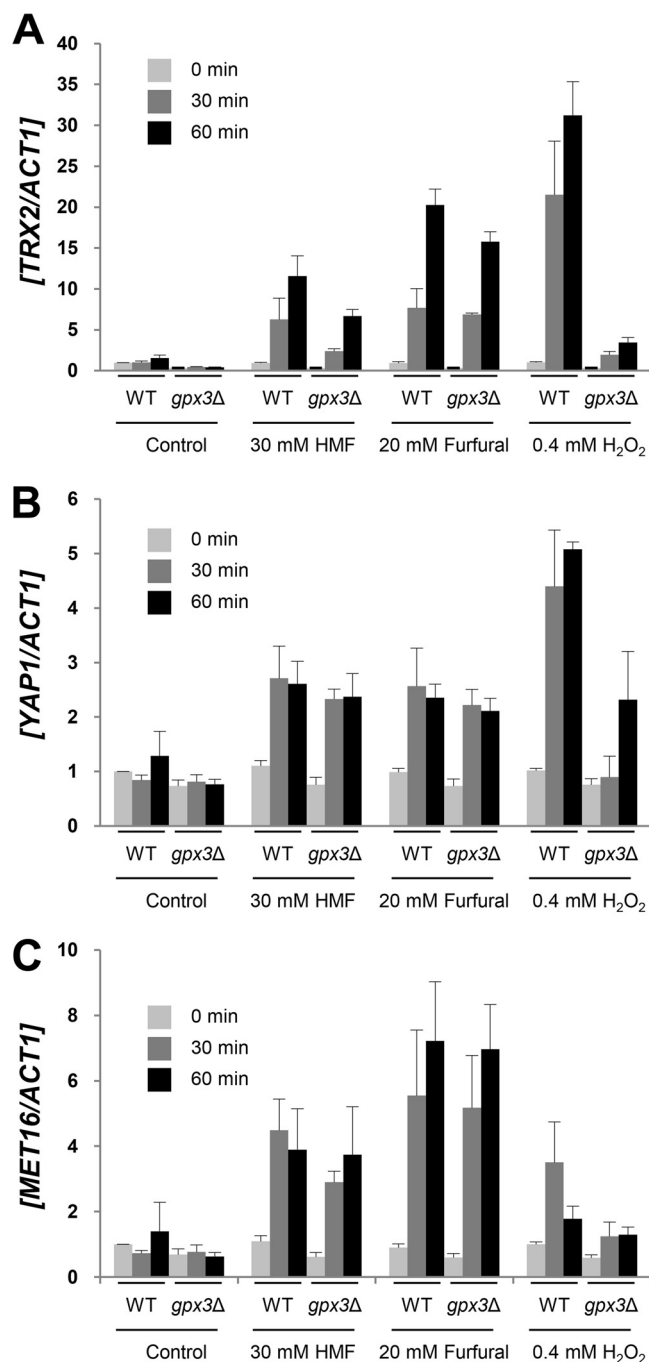

FIG 2 Furfural and HMF activate Yap1 as thiol-reactive electrophiles. BY4741 (wild-type [WT]) and *gpx3Δ* cells were grown in YPD medium until early exponential phase and then treated with the indicated amounts of HMF, furfural, and H_2O_2 for 30 or 60 min. The mRNA expression levels of *TRX2* (A), *YAP1* (B), and *MET16* (C) were detected by qRT-PCR and normalized to *ACT1*. The mRNA levels relative to those of untreated wild-type control were indicated with standard deviations of three independent experiments.

as a redox buffer and plays a central role in cellular protection against oxidative stress (30). To investigate the effects of furfural and HMF on GSH, we incubated HMF or furfural with 5 mM GSH and detected the reactivity by measuring the decrease in thiol concentration of GSH. After 15 min of incubation, GSH concen-
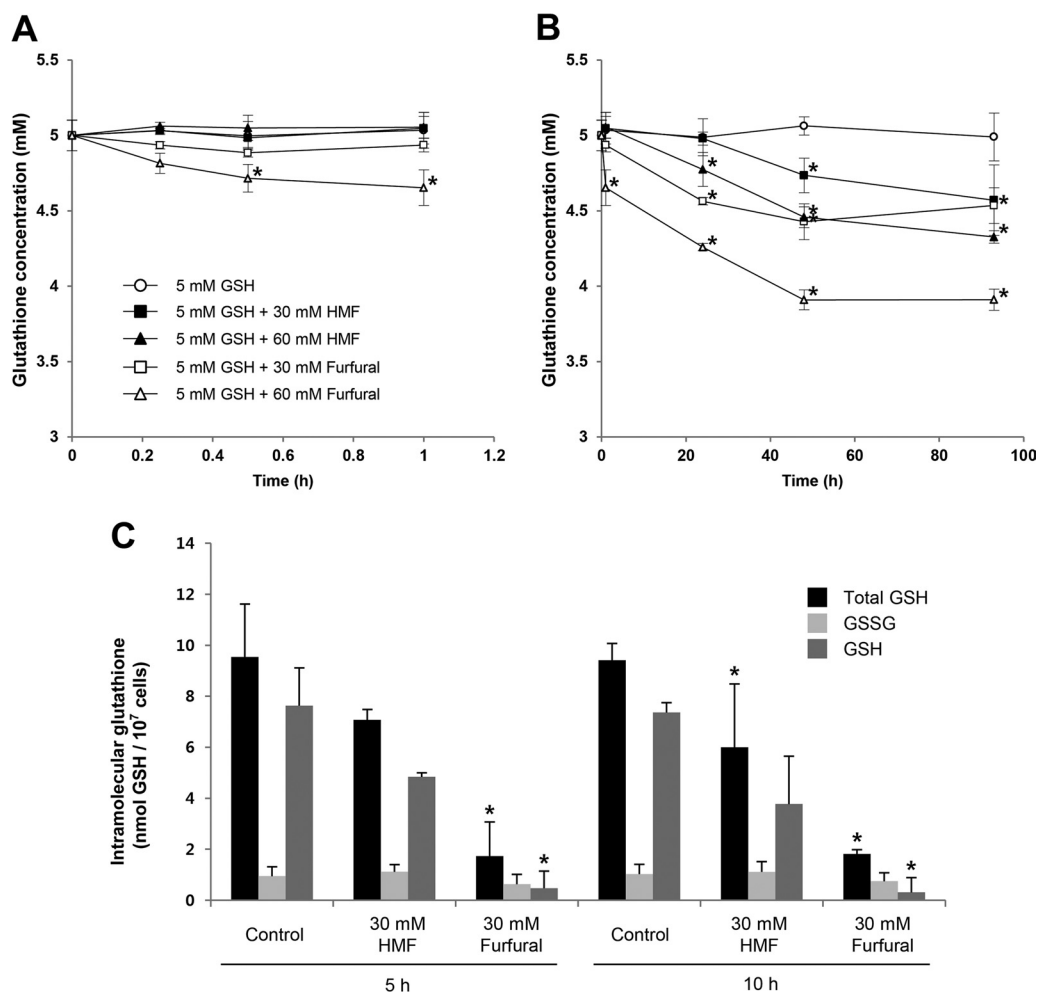
FIG 3 Furfural shows higher reactivity than does HMF toward GSH *in vitro* and *in vivo*. (A and B) The indicated concentrations of furfural or HMF were incubated with 5 mM GSH at 30°C, and the concentrations of GSH sulfhydryl groups were measured up to 1 h (A) and up to 96 h (B). Error bars indicate standard deviations of three independent experiments. *, significantly lower than the 5 mM GSH control (*t* test; $P < 0.05$). (C) Early-exponential-phase cells grown in YPD medium were treated with 30 mM HMF or furfural for 5 h and 10 h. Deproteinated cell lysates were assayed for the measurement of total GSH and GSSG levels, and GSH levels were calculated by abstracting the amounts of GSSG from those of total GSH. Error bars indicate standard deviations of three independent experiments.*, significantly lower than the control (*t* test; $P < 0.05$).

trations were reduced to 4.93 mM and 4.82 mM in the presence of 30 mM and 60 mM furfural, respectively (Fig. 3A). However, no significant reduction of GSH concentrations was detected in the presence of HMF for up to 1 h, suggesting higher reactivity of furfural than of HMF toward GSH (Fig. 3A). Upon longer incubation of up to 96 h, an HMF-dependent reduction of GSH concentration was also observed (Fig. 3B). The higher thiol reactivity of furfural than of HMF agrees with the predicted electrophilicity of these compounds. HMF has an additional hydroxymethyl group, which acts as an electron-donating group reducing electrophilicity.

We also investigated the effects of furfural and HMF on GSH and GSSG levels *in vivo* (Fig. 3C). In agreement with the fact that most of the cellular glutathione is present in a reduced form, the untreated control cells showed a GSH/GSSG ratio of 8:1. Treatment with 30 mM HMF reduced the GSH levels by 37% to 49% without affecting the GSSG levels, resulting in GSH/GSSG ratios of 4.3:1 and 3.4:1 after 5 and 10 h, respectively. A 30 mM concentration of furfural exerted more dramatic depletion of GSH to

levels even lower than those of GSSG, resulting in GSH/GSSG ratios of 0.74:1 and 0.41:1 after 5 and 10 h, respectively. These results suggest that although both furfural and HMF reduce cellular GSH levels possibly by direct interaction, furfural is much more effective in GSH depletion.

Overexpression of an active *YAP1* mutant increases tolerance to furfural and HMF. Since both furfural and HMF act as thiol-reactive electrophiles which can induce oxidative stress, the overexpression of *YAP1* might confer resistance to furfural and HMF by activation of genes involved in resistance to oxidative stress and reduction of furan aldehydes (17, 31). Indeed, overexpression of *YAP1* has been shown to convey resistance to HMF, although the detailed mechanisms have not been investigated (18).

Based on our observation that furfural can also activate Yap1, we examined whether overexpression of *YAP1* can also increase tolerance to furfural. In addition to wild-type *YAP1*, we overexpressed the *YAP1^{C620F}* mutant under an *ADH1* promoter and examined cell growth in the presence of furfural or HMF. *YAP1^{C620F}*
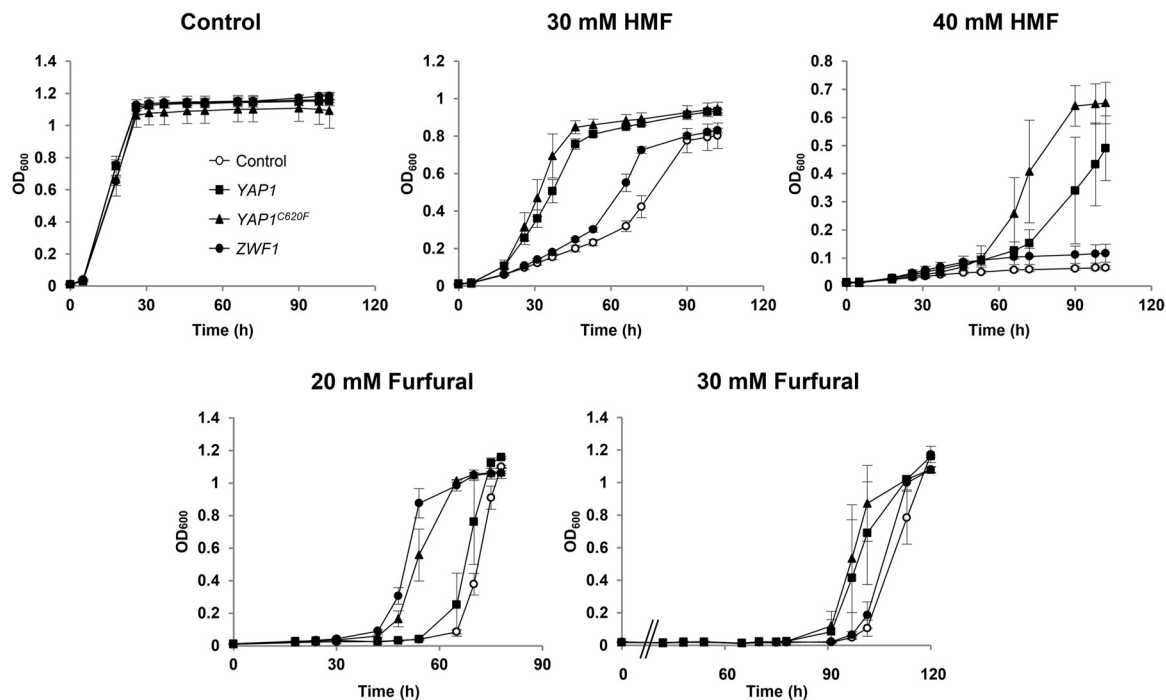
FIG 4 Overexpression of *YAP1* or *YAP1*[C620F] increases tolerance to HMF and furfural. Cells overexpressing *YAP1*, *YAP1*[C620F], or *ZWF1* were grown in SC-Leu medium containing 30 mM or 40 mM HMF or 20 mM or 30 mM furfural. Cells containing pRS415ADH empty vector were used as a control. All experiments were performed in triplicate, and error bars indicate standard deviations. OD_{600}, optical density at 600 nm.

is a gain-of-function allele of *YAP1*, originally isolated as a mutant conferring resistance to diazaborine (32). *ZWF1*, involved in NADPH production through the pentose phosphate pathway, was also overexpressed as a positive control (15). As shown in Fig. 4, cells overexpressing *ZWF1* showed slightly better growth than did the control in the presence of 30 mM or 40 mM HMF. However, overexpression of *YAP1* was more efficient than *ZWF1* overexpression for enhancing the HMF resistance. Furthermore, *YAP1*[C620F]-overexpressing cells showed higher HMF tolerance than did *YAP1*-overexpressing cells, reflecting the higher activity of the Yap1[C620F] mutant.

Although overexpression of *YAP1*, *YAP1*[C620F], and *ZWF1* also increased tolerance to furfural, the effects of each gene slightly differed depending on the furfural concentration (Fig. 4). In the presence of 20 mM furfural, cells overexpressing *ZWF1* showed better growth than did cells overexpressing *YAP1*[C620F] or *YAP1*, which might reflect a major role of NADPH depletion for the furfural toxicity at this concentration. However, at a higher concentration of 30 mM, the overexpression of *YAP1*[C620F] or *YAP1* was more efficient than the overexpression of *ZWF1* in increasing furfural tolerance.

Overexpression of catalase increases furfural and HMF tolerance. Although H_2O_2 does not seem to play a major role in the activation of Yap1 in the presence of furfural or HMF (Fig. 2), the accumulated H_2O_2 could be one of the mediators exerting the toxicity of furfural and HMF. To test this possibility, we overexpressed catalases that decompose H_2O_2, and examined the effects on furfural and HMF tolerance. *CTA1* encoding mitochondrial catalase and *CTT1* encoding cytosolic catalase are known targets of Yap1 (29). Overexpression of *CTA1* and *CTT1* commonly led to furfural and HMF tolerance, indicating that H_2O_2 indeed plays

a part in the toxicity of furfural and HMF (Fig. 5A). For HMF tolerance, *CTA1* was more effective than *CTT1*.

Increase in cellular GSH levels enhances tolerance to furfural but not to HMF. Since furfural and HMF react with cellular GSH (Fig. 3), GSH might play a protective role against furfural and HMF. We investigated this possibility by overexpressing *GSH1* and *GSH2*, involved in glutathione biosynthesis, and *GLR1*, encoding glutathione reductase that recycles the oxidized GSSG back to GSH (33). The first and rate-limiting step of GSH biosynthesis is the production of γ-glutamyl cysteine (γ-GC) by Gsh1, and Gsh2 catalyzes the production of GSH from γ-GC and glycine (33). The expression of *GSH1*, *GSH2*, and *GLR1* genes is all regulated by Yap1 (34, 35). As shown in Fig. 5B, the overexpression of *GSH1* and *GLR1*, but not *GSH2*, increased cellular tolerance to furfural, suggesting the protective effect of GSH against furfural toxicity. However, neither *GSH1* nor *GSH2* overexpression increased tolerance to HMF, while *GLR1* overexpression slightly increased the tolerance (Fig. 5B). Therefore, the protective role of GSH seems to be specific to furfural, which is more effective than HMF in GSH depletion.

Addition of GSH and dithiothreitol (DTT) to the medium reduces toxicity of furfural but not HMF. Next, we examined whether GSH added to the medium could also elicit protective effects against furfural and HMF. In accordance with the effect of GSH overexpression *in vivo*, the addition of GSH in rich YPD medium exerted a protective effect on cell growth only in the presence of furfural but not HMF. Although furfural tolerance was increased by the addition of GSH in a dose-dependent manner, the cells became more sensitive to HMF in the presence of GSH (Fig. 6A).

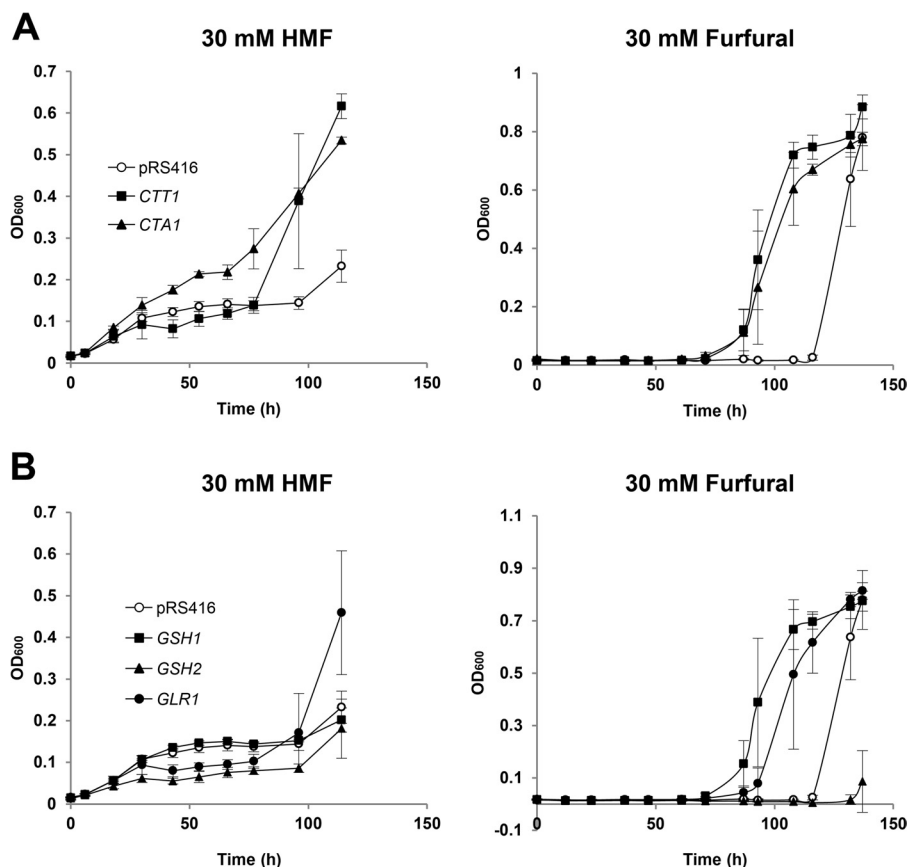We also tested the effects of DTT on furfural and HMF toler-

FIG 5 Effects of overexpression of antioxidant genes on tolerance to furfural and HMF. Cells overexpressing *CTA1* or *CTT1* (A) or *GSH1*, *GSH2*, or *GLR1* (B) were grown in SC-Ura medium containing 30 mM HMF or furfural. Cells harboring pRS416ADH vector were used as a control. All experiments were performed in triplicate, and error bars indicate standard deviations.

ance. DTT is a cell-permeant reducing agent that can prevent disulfide bond formation (36). Treatment with DTT has been shown to induce reductive stress (37). Accordingly, cell growth was slightly reduced in the presence of DTT in a dose-dependent manner (Fig. 6B). Even with such a growth-inhibitory effect, DTT facilitated cell growth in the presence of furfural, suggesting that thiol oxidation is one of the toxic effects of furfural. In contrast, DTT exerted a negative effect on cell growth in the presence of HMF (Fig. 6B).

To examine whether the effects of GSH and DTT on furfural and HMF tolerance could be dependent on culture medium, we also tested the effects of GSH and DTT in synthetic complete (SC) medium, where furfural and HMF show higher toxicity than in YPD medium. As shown in the YPD medium, both GSH and DTT showed protective effects against furfural while inhibiting cell growth in the presence of HMF in SC medium (Fig. 6C).

Next, we investigated whether the protective role of GSH against furfural requires transport of GSH inside the cell. Hgt1 is a high-affinity GSH transporter, and an *hgt1Δ* deletion mutant showed no detectable plasma membrane GSH transport (38). Therefore, we compared the protective effects of GSH in wild-type and *hgt1Δ* strains. In the presence of furfural, the *HGT1* deletion mutant showed a longer lag phase than did the wild type, implying that GSH uptake from the medium has an advantage in furfural tolerance (Fig. 6D). However, when 5 mM GSH was provided in

the medium, the *hgt1Δ* strain as well as the wild type showed increased furfural tolerance, indicating that GSH can play a protective role against furfural even outside the cell. The protective role of extracellular GSH against furfural might be the result of the GSH-dependent protection of surface-exposed thiols. In addition, GSH could directly react with furfural, reducing the effective concentration of furfural.

DISCUSSION

Furfural and HMF are known to induce oxidative stress, but the underlying mechanisms have not yet been clarified. In this study, we demonstrated that furfural and HMF act as thiol-reactive electrophiles, thus activating the Yap1 transcription factor and reducing cellular GSH levels. In addition to the consumption of NADPH during the reductive detoxification of furfural and HMF, such thiol reactivity might be responsible for the induction of oxidative stress and ROS accumulation by these compounds.

Covalent modification or oxidation of sulfhydryl groups can affect the structure and function of enzymes and other proteins with various biological functions. The redox status of protein sulfhydryl groups is mainly regulated by glutaredoxins (GRXs) and thioredoxins (TRXs), which are reduced by GSH- and NADPH-dependent thioredoxin reductase, respectively (33). The oxidized GSSG is reduced by glutathione reductase, using NADPH as a reducing power (35). GSH also acts as a reductant for glutathione
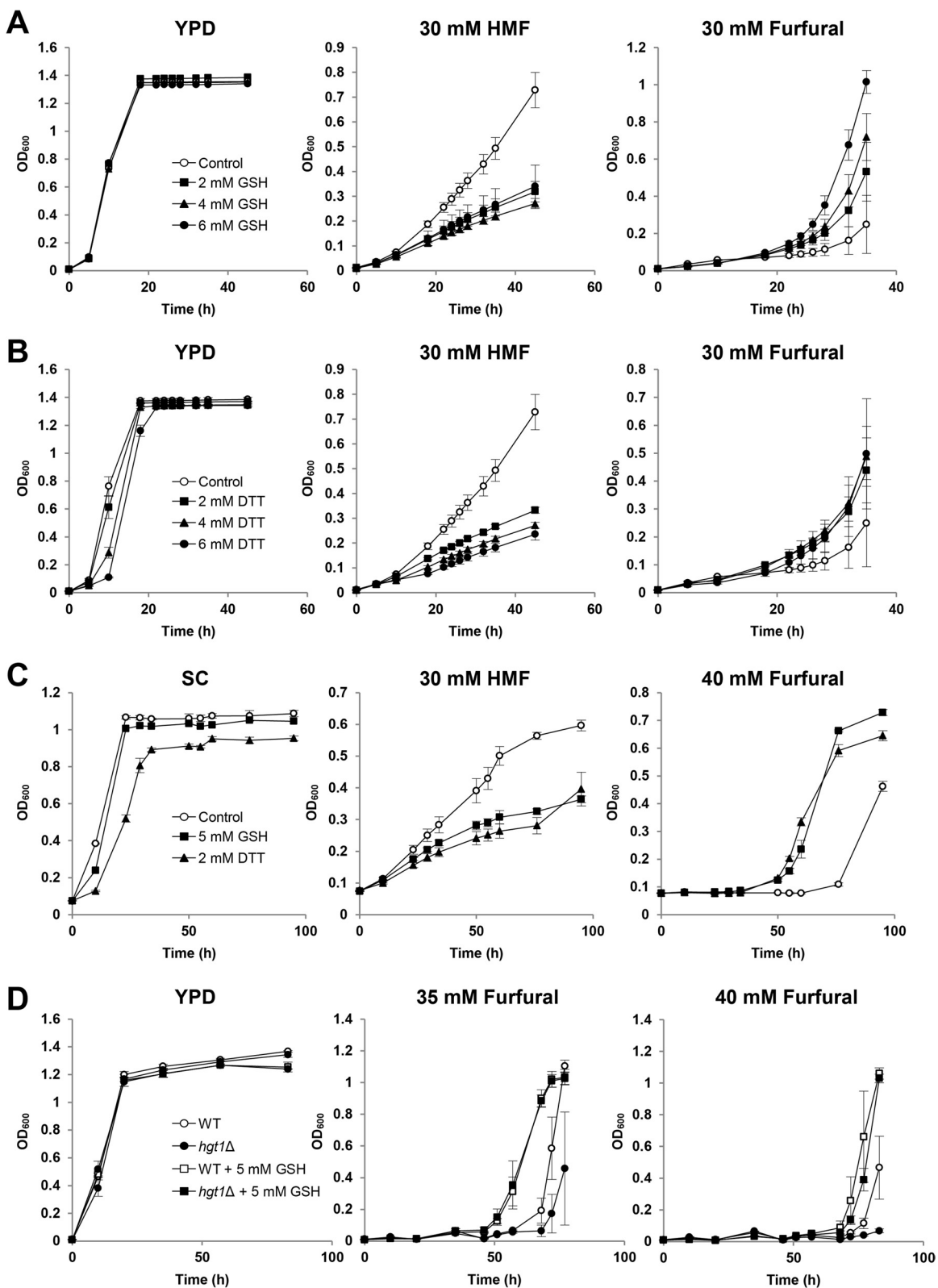
FIG 6 Addition of GSH or DTT to medium increases tolerance to furfural. (A and B) Cells were grown in YPD medium containing 30 mM HMF or furfural in the presence of the indicated concentrations of GSH (A) or DTT (B). (C) Cells were grown in SC medium containing 30 mM HMF or 40 mM furfural in the presence of 5 mM GSH or 2 mM DTT. (D) Wild-type and *hgt1Δ* cells were grown in YPD medium containing 35 mM or 40 mM furfural in the absence or presence of 5 mM GSH. All experiments were performed in triplicate, and error bars indicate standard deviations.

peroxidase involved in the reduction of H_2O_2 and organic hydroperoxides (39). Therefore, maintenance of GSH and NADPH levels is important for cellular protection against oxidative stress.

We have shown that furfural has higher thiol reactivity than does HMF, thus more effectively depleting GSH levels *in vitro* and *in vivo*. Cellular GSH levels were reduced by 16-fold with a 5-h treatment with 30 mM furfural but by only 1.6-fold with 30 mM HMF. Such higher thiol reactivity might be partly responsible for the higher toxicity of furfural than of HMF. Considering the central role of GSH in cellular protection against oxidative stress (30), the depletion of GSH might be one of the major toxic mechanisms of furfural. In support of this notion, the overexpression of genes for GSH synthesis (*GSH1*) and GSSG reduction (*GLR1*), or addition of GSH in the medium, conferred resistance to furfural. The *hgt1Δ* strain, which cannot take up GSH, was more sensitive to furfural than was the wild type but still showed increased furfural resistance in the presence of extracellular GSH. Therefore, GSH seems to play a protective role against furfural both inside and outside the cell. In addition to its role against oxidative stress, GSH is also involved in the detoxification of electrophilic xenobiotics through the formation of GSH S-conjugates, which are subsequently transported into the vacuole or out of the cell (40). Therefore, we cannot rule out the possibility that GSH might also be involved in the detoxification of furfural by this pathway.

Although HMF could also reduce cellular GSH levels, HMF tolerance was largely unaffected by increasing expression of genes for GSH biosynthesis. Therefore, the relatively mild reduction in GSH levels seems not to be critical for the toxicity of HMF. Considering the fact that HMF has weaker thiol reactivity than does furfural, other effects such as NADPH depletion, ROS accumulation, and modification of other cellular targets might play larger roles for HMF toxicity. Unexpectedly, the addition of GSH or DTT in the medium further inhibited cell growth in the presence of HMF. Further studies are needed to elucidate the mechanisms for the negative effect of GSH and DTT on HMF tolerance.

Although furfural and HMF commonly act as thiol-reactive reagents inducing oxidative stress, their differences in reactivity might affect a different range of cellular targets, thus exhibiting differential toxic mechanisms. Therefore, pleiotropic gene expression by overexpression of $YAP1^{C620F}$ might be an effective strategy to generate a furfural- and HMF-tolerant yeast strain. Overexpression of *YAP1* might confer resistance to furfural and/or HMF by activation of genes for antioxidant enzymes, including *CTA1*, *CTT1*, *GSH1*, and *GLR1* as demonstrated in this study. Furthermore, Yap1 targets also include *ADH7* and *GRE2*, which are involved in the reduction of furan aldehydes (17, 31). Yap1 also regulates the expression of efflux pumps, including *FLR1*, *YCF1*, and *SNQ2*, which might be implicated in the detoxification of furfural and/or HMF (17, 32). The furfural and HMF tolerance could be further increased by optimizing $YAP1^{C620F}$ expression levels and by screening more *YAP1* mutants with enhanced activity.

Taken together, for the first time we elucidated the thiol reactivity of furfural and HMF as the underlying mechanisms by which oxidative stress is induced by these compounds. Furthermore, we provided new evidence that furfural and HMF might cause differential cytotoxic effects due to the differences in thiol reactivity. The similarities and differences in toxicity between furfural and HMF might provide useful information for the future development of tolerant strains for cellulosic ethanol production.

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